Agrobacterium–mediated Transformation and Regeneration of Fertile Transgenic Plants of Rapeseed (*Brassica napus L.*)

P. Jonoubi[1, 2], A. Mousavi[1], A. Majd[2], M. Jalali-Javaran[3], J. Daneshian[4] and A. H. Salmanian[1]

1-National Research Center for Genetic Engineering and Biotechnology, Tehran, Iran.
2-Teacher Training University, Tehran, Iran. pjonoubi@hotmail.com
3-Tarbiat Modarres University, Tehran, Iran.
4-Seed and Plant Improvement Institute, Oilseeds Research Department, Karaj, Iran.

Abstract
A method for transformation of *B.napus* is presented in this study. The protocol is based on infection of cotyledonary petioles of 5-day-old and hypocotyl segments of 14-day-old seedlings with an *Agrobacterium tumefaciens* strain LBA 4404 carrying a disarmed binary vector pBI121. The T-DNA region of this binary vector contains the neomycin phosphotransferase II (*NPT*II) and glucuronidase (*GUS*) genes. After cocultivation for 48h, the explants were placed on shoot induction media containing 15 mgl^{-1} kanamycine sulfate. Shoot induction was continued 4-6 weeks. After 2 weeks, the green shoots were transferred to root induction medium. After 4-8 weeks rooted plantlets were transferred to greenhouse. 16 kanamycine resistance plants from 244 cotyledonary petioles for SLM and 20 transformed plants from 151 explants for PF and 6 kanamycine resistance plant from 75 hypocotyls were obtained for both varieties. Most of them grew to maturity, produced normal flowers and set seeds. *GUS* assay and polymerase chain reaction (PCR) confirmed the introduction of the T-DNA into the rapeseed genome.

Keywords: *GUS* assay- kanamycine resistance plant- cotyledon- hypocotyl

Introduction
Rapeseed is an important edible and industrial oil seed crop worldwide. It is also becoming popular oilseed. Cropland area under cultivation has increased from less than 100 ha to more than 50000 ha during lost live years in Iran.
Recent advances in cell and molecular biology have facilitated the transfer of foreign genes into plants, which is the first step toward the genetic improvement of crops using the biotechnological approach. Several different techniques such as microinjection, electroporation, direct plasmid uptake, direct DNA uptake for transferring DNA into Brassica species have been used. *Agrobacterium*-mediated transformation has proven to be the most efficient method (De Block et al., 1989; Moloney et al., 1989; Mukhopadhyay, et al., 1991; Schroder et al. 1994; Jun et al.,1995; Christey et al., 1999).
As part of a study to produce transgenic plant of *B.napus*, we have developed methods for the regeneration of plants from cotyleclonary petioles and hypocotyl segments. In this study, we report an efficient gene transfer system for the production of normal, fertile transgenic plant of *B.napus* using *Agrobacterium tumefaciens*- mediated transformation.

Materials and Methods
Plant materials
Seeds of *B.napus* varieties PF 7045/91 and SLM 046 were surface sterilized in 1.5% sodium hypochlorite and 0.6% Triton 20 for 10 min. The seeds were washed in sterile distilled water 5 times and planted on germination medium containing 50% MS medium. Seeds were germinated at 24ºC in a 16h light/8h dark. After 5 days the cotyledons were used. Hypocotyls were excised from 14 days old seedling.

Transformation procedure
Single colonies of *Agrobacterium tumefaciens* strains EHA 101 and LBA 4404 carrying a binary vector PBI121 were grown overnight at 28ºC in LB medium supplemented with 50 mgl^{-1} kanamycine. The binary vector PBI121 contains a CaMV35S promoter, *NPTII* and *GUS* genes.
The bacterial culture was diluted 1:2 with liquid *Agrobacterium* Infection medium (MS medium containing 1% glucose pH: 5.2). This suspension was used as the bacterial inoculum. The cut

surface of the excised cotyledons was immersed into this bacterial suspension for a few seconds. They were immediately returned to the shoot induction medium (SIM) containing 4.5 $mg1^{-1}$ benzyladenine (BA). The cotyledons were co-cultivated with the Agrobacterium for 48h. The cotyledons were transferred to SIM supplemented with 200 mgl^{-1} cefotaxime and 15 mgl^{-1} Kanamycine sulfate. Sections were subsequently transferred to a fresh medium of the same composition every two weeks. After 6 weeks the green shoots were transferred to root induction medium (RIM) Containing $2mgl^{-1}$ indole butyric acid and 150 mgl^{-1} cefotaxime.
The hypocotyl sections were inoculated with *Agrobacterium* by immersing for 10 min into the bacterial inoculum. Then, they were returned to the cocultivation medium containing $1mgl^{-1}$ 2,4-D, pH: 5.2 at 25ºC for 48h in dark. After the cocultivation, The hypocotyl sections were transferred to a callus induction medium (B5 medium, $1mgl^{-1}$ 2,4-D, pH: 5.8) supplemented with 200 mgl^{-1} cefotaxime and cultured for 7 days. Then, They were transferred to SIM containing 3 mgl^{-1} BA and 0.3 mgl^{-1} thidiazuron (TDZ) supplemented with 200 mgl^{-1} cefotaxime and 10 mgl^{-1} kanamycine. Sections were subsequently transferred to a fresh medium of the same composition every 2 weeks to obtain regenerated shoots. After 6 weeks, green shoots were excised from calli and placed on RIM Supplemented with 150 mgl^{-1} cefotaxime. After 4-5 weeks rooted plantlets were transferred to soil. Plants were grown to maturity and produced set seeds.

Histochemical *GUS* assay

Histochemical *GUS* expression of petiole and leaf segments of green plant was assayed. These tissues were dipped in a *GUS* assay buffer containing 50mM phosphate buffer (pH: 7.0), 1mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 1mM EDTA, 0.001% Triton X-100 and 10mM β-mercaptoethanol incubated overnight at 37ºC. Chlorophyll of leaf segments was destained by rinsing them with 96% ethanol.

DNA extraction and PCR analysis

Total DNA was extracted from young leaves of green plants by the cetyltrimethylammonium bromide (CTAB) method. Four specific oligonucleotides derived from *NPTII* and *GUS* genes were used as primers for PCR. For amplification of a 500 bp fragment of the *GUS* gene, the primers used, were:
5´-CCG GCA TAG TTA AAG AAA TCA T and 5´-TGG TCA GTC CCT TAT GTT ACG. For amplification of a 1200 bp fragment of *NPTII* gene, the primes used, were: 5´- TTG AAC GAT CGT CGG GGA TCA and 5´- GTC GCC TAA GGT CAC TAT CAG.
One µl of DNA was used in a 20 µl PCR reaction containing 3mM MgCl2, 250 µM dNTPs, 0.25 µM of each primer, 1x PCR buffer, 0.5 unit of Taq polymerase (Cinagene) and 11.5 µl ddH_2O. PCR Conditions: 94ºC for 1min, 56ºC for *GUS* gene and 58ºC for *NPTII* gene for 1min and 72ºC for 1 min for 30 cycles in Perkin Elmer thermocycler. 8µl of PCR reaction was run on a 1% agarose gel and visualized by ethidium bromide staining.

Results

In the cotyledonary petioles, cells around the cut end of petioles are an ideal target for *Agrobacterium*-mediated transformation as the cells undergoing organogenesis are those most readily accessed by the *Agrobacterium* treatment (Moloney et al. 1989).The first transformed shoots developed after about 4 weeks on SIM. Most green plants produced roots after 4 to 8 weeks. The rooted shoots were placed on soil and produced fertile flower and set seeds.
EHA101 strain activated very aggressively and cefotaxime couldn't control it in media. Then, LBA4404 strain was used for infecting of explants and transformation. For selection of transformed from untransformed shoots, kanamycine were used as selectable marker. The *NPTII* gene has been the most widely used marker gene for selection of transformation. The transformed shoots containing *NPTII* gene had a normal dark green color and quiet vigorous growth in selection medium supplemented with kanamycine. Although the untransformed shoots appeared as pale green leaflets with severe anthocyanin coloration on their surface.
The result showed that 16 kanamycine resistance plants from 244 cotyledonary petioles for SLM and 20 transformed plants from 151 explants for PF var. were obtained. Hypocoyl explants were produced calli on CIM. Green calli were initiated shoots on SIM. Green shoots were cut from calli and transferred to RIM. Roots were formed in Kanamycine resistance plants after 4-8 weeks. Rooted plantlets were transferred to soil and produced fertile flowers and set seeds. Six kanamycine resistance plants from 75 hypocotyls were obtained for both varieties.

GUS gene expression in kanamycine resistant plants was investigated. The majority of transgenic plants (70%) were found to express *GUS* gene in the leaves and petioles. *GUS* activity was mostly detected in the vascular tissues. PCR analysis using DNA from kanamycine resistance plants confirmed that most of green plants contained *GUS* and *NPTII* genes.

Discussion

In transformation in *B. napus,* some green shoot buds could differentiate on calli from inoculated hypocotyls and cotyledons on selective medium at the initial stage of selection, but most of them were later bleached and eliminated in the further selection. A small number of cells on inoculated explants were transformed after co-cultivation with *A. tumefaciens.*

Vitrification posed a problem in Brassica tissue culture. Vitrified shoots were difficult to root formation and often died shortly after their transfer to greenhouse. The results showed that decreasing of sucrose on RIM diminished vitrification and stimulated root formation. De Blocke et al. (1989) found that a lowing of sucrose concentration or using glucose instead of sucrose decreased vitrification.

The cells of cotyledonary cut surface are accessible to *Agrobacterium.* In our experiments frequencies of transformation with cotyledonary explants were 6.5% for SLM and 13.3% for PF. While Moloney et al. (1989) obtained 22.4% frequency of transformation. Li et al. (1995) produced 2.9% and Bar Field and Pua (1991) obtained 9% in capacity of transformation from cotyledonary petioles. We can conclude that this procedure produced high efficiency for *Agrobacterium* transformation.

Our results show that *B. napus* hypocotyl tissue is also amenable to genetic transformation. We obtained 8% frequency of transformation from hypocotyl explants. Schroder et al. (1994) reported 10% transformation efficiency. Radke et al. (1992) and Takasaki et al. (1997) obtained 2.2% and 5% transformed plants.

The transfer system described in this method has a great potential for genetic improvement of *B.napus* by introducing genes responsible for agronomically important traits, i.e. herbicide tolerance and insect resistance into the plants.

References

Christey, M. C., R. H. Braun, F. O. Kenel and E. Podivinsky.1999. *Agrobacterium*-mediated transformation of swede (*Brassica napus* L.). Proc. 10th Int. Rapeseed Congr., Canberra, Australia.

De Block, M., D. De Brouwer and P. Tenning.1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol.91: 694-701.

Eapen, S., S. Tivarekar and L. George. 1998. Thidiazuron-induced shoot regeneration in pigeonpea (*Cajanus cajan* L.). Plant Cell Tiss. and Org. Cult.53: 217-220.

Jun, S.L., S. Y. Kwon, K. Y. Peak and K. H. Peak.1995. *Agrobacterium*-mediated transformation and regeneration of fertile transgenic plants of chinese cabbage (*Brassica compesteris ssp. pekenensis* cv. spring flavor). Plant Cell Rep.14: 620-625.

Li, X.B., H.Z. Mao and Y.Y. Bai. 1995. Transgenic plants of rutabaga (*Brassica napobrassica*) tolerant to pest insects. Plant Cell Rep.15: 97-101.

Moloney, M.M., J. M. Walker and K. Sharma.1989. High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep.8: 238-242.

Mukhopadhyay, A., R. Topfer, A. K. Pradhan, Y. S. Sohdi, H. H. Steinbil, J. Schell and D. Pental.1991. Efficient regeneration of *Brassica oleracea* hypocotyl protoplast and high frequency genetic transformation by direct DNA uptake. Plant Cell Rep.10: 375-379.

Radke, S.E., J. C. Turner and D. Facciotti.1992. Transformation and regeneration of *Brassica rapa* using *Agrobacterium tumefaciens*. Plant Cell Rep.11: 499-505.

Schroder, M., C. Dixelius, L. Rahlen and K. Glimelius.1994. Transformation of *Brassica napus* by using the *aadA* gene as selectable marker and inheritance studies of the marker genes. Physiol. Plant.92: 37-46.

Takasaki, T., K. Hatakeyama, K. Ojima, M. Watanabe, K. Toriyama and K. Hinata.1997. Factors influencing *Agrobacterium*-mediated transformation of *Brassica rapa* L. Breed. Sci.47: 127-134.